KPC-50 confers resistance to ceftazidime-avibactam associated with reduced carbapenemase activity

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Abstract: KPC-50 is a KPC-3 variant identified from a Klebsiella pneumoniae clinical isolate recovered in Switzerland in 2019. As compared to KPC-3, KPC-50 shows i) a three amino-acid insertion (Glu-Ala-Val) between amino acids 276 and 277 amino acid sequence, (ii) an increased affinity to ceftazidime, (iii) a decreased sensitivity to avibactam, explaining the ceftazidime-avibactam resistance, and (iv) associated to a sharp reduction of its carbapenemase activity.

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KPC-50 confers resistance to ceftazidime-avibactam associated with reduced carbapenemase activity

Laurent Poirel[a,b,c*], Xavier Vuillemin[a], Mario Juhas,[a] Amandine Masseron[a], Ursina Bechtel-Grosch[d], Simon Tiziani,[e] Stefano Mancini,[f] and Patrice Nordmann[a,b,c,g]

[a]Emerging Antibiotic Resistance Unit and Medical and Molecular Microbiology, Faculty of Science and Medicine, University of Fribourg, Fribourg, Switzerland, [b]Swiss National Reference Center for Emerging Antibiotic Resistance (NARA), University of Fribourg, Fribourg, Switzerland, [c]INSERM European Unit (IAME, France), University of Fribourg, Fribourg, Switzerland, [d]Department of Trauma Surgery, University Hospital of Zürich, Zürich, Switzerland, [e]Institute of Intensive Medicine, University Hospital Zürich, University of Zürich, Zürich, Switzerland, [f]Institute of Medical Microbiology, University of Zürich, Zürich, Switzerland, and [g]Institut for Microbiology, University Hospital Center and University of Lausanne, Lausanne, Switzerland

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***Address correspondence to:**
Laurent Poirel
Medical and Molecular Microbiology
Faculty of Science and Medicine, University of Fribourg
Chemin du Musée 18

CH 1700 Fribourg, Switzerland
Phone: +41 26 300 9582
E-mail: laurent.poirel@unifr.ch

KPC-50 is a KPC-3 variant identified from a *Klebsiella pneumoniae* clinical isolate recovered in Switzerland in 2019. As compared to KPC-3, KPC-50 shows i) a three amino-acid insertion (Glu-Ala-Val) between amino acids 276 and 277 amino acid sequence, (ii) an increased affinity to ceftazidime, (iii) a decreased sensitivity to avibactam, explaining the ceftazidime-avibactam resistance, and (iv) associated to a sharp reduction of its carbapenemase activity.

Occurrence of multidrug-resistant Enterobacterales, and especially of carbapenemase-producing isolates, is increasingly reported, thus leaving very few therapeutic options for treating related infections (1). Interestingly, the recently-marketed ceftazidime/avibactam (CZA) drug combination offers novel interesting perspectives (2). This β-lactam/β-lactamase inhibitor combo provides a therapeutic alternative for treating infections caused by KPC-like and OXA-48-like producers, whereas producers of carbapenemases of the metallo-ß-lactamase type remain resistant to that combination (1, 2). Despite CZA is still rarely prescribed worldwide, KPC-like producing isolates being resistant to this drug combo have been already reported (3-7). Several reports identified KPC variants exhibiting single amino acid substitutions in their omega-loop (amino acid positions 164-179), and particularly the Asp179Tyr substitution, leading to enhanced affinity toward ceftazidime, with a concomitant reduced binding to avibactam (8-12). In addition, we recently identified KPC-41, possessing a three amino-acid insertion in the KPC-3 protein sequence being distantly located from the omega loop (namely between positions 269-270), that conferred high level of resistance to CZA in a clinical *K. pneumoniae* isolate recovered in Switzerland (13).

K. pneumoniae isolate N869 was recovered from a patient repatriated after a traffic accident from Greece to Switzerland. In the Greek hospital, the patient developed a ventilator-associated pneumonia for which he received a treatment made of clindamycin, linezolid and meropenem during two days, to which colistin was added on day 5. Few days later, he was transferred to Switzerland where all antibiotics but meropenem (as monotherapy then) were discontinued. Rectal swabs performed at admission grew *K. pneumoniae* isolate N859 using the Chrom ID Carba Smart® selective plate (bioMérieux, La Balme-les-Grottes, France). According to the EUCAST 2020 breakpoints (14), *K. pneumoniae* isolate N859 was resistant to all ß-lactams including to imipenem and ertapenem, but remained susceptible to meropenem (Table 1). The carbapenemase activity was evaluated by using the Rapid Carba NP test that gave a positive result (15).

K. pneumoniae N859 was also resistant to aminoglycosides (kanamycin, tobramycin, netilmicin), to fluoroquinolones and to colistin (MIC at 128 µg/ml). It remained susceptible to tetracycline, tigecycline, chloramphenicol, trimethoprim-sulfamethoxazole and fosfomycin, and was of intermediate susceptibility to amikacin and gentamicin. It also showed resistance to CZA (MIC at >256 µg/ml) and to ceftolozane-tazobactam combination (> 256 µg/ml), using inhibitors concentrations at 4 µg/ml.

74 PCR identified a *bla*$_{KPC}$-like gene and sequencing of the corresponding amplicon
75 identified a gene encoding a KPC variant possessing a three amino-acid insertion (Glu-Ala-
76 Val) between amino acids 276 and 277 (Ambler numbering), leading to a novel variant named
77 KPC-50 (Figure S1). Search of additional ß-lactamase resistance genes as reported (16)
78 identified a *bla*$_{SHV}$-like gene (intrinsic to *K. pneumoniae*) but no additional extended-spectrum
79 ß-lactamase gene. Mating-out assays performed using *K. pneumoniae* N859 as donor and
80 azide-resistant *Escherichia coli* J53 strain as recipient (13) were successful and confirmed the
81 plasmid location of the *bla*$_{KPC-50}$ gene, being ca. 60-kb in-size (data not shown). No other
82 antibiotic marker was co-transferred along with the *bla*$_{KPC-50}$ gene. PCR-based replicon typing
83 showed that this plasmid belonged to the IncFIB incompatibility group (17). Multilocus
84 sequence typing performed as described (18) showed that isolate N859 belonged to sequence
85 type ST258 that corresponds to the worldwide spread KPC-producing *K. pneumoniae*
86 background (19, 20).

87 In order to confirm if the amino acid substitutions identified within the KPC sequence
88 might be responsible for the CZA resistance phenotype observed in *K. pneumoniae* N859, the
89 *bla*$_{KPC-50}$ gene was cloned and expressed in *E. coli* TOP10. MIC values were then compared
90 with the previously obtained KPC-3-producing *E. coli* TOP10 (13). In such *E. coli*

91 background, KPC-3 conferred resistance to all β-lactams including ceftazidime, but remained
92 susceptible to CZA, as previously shown (13). Conversely, although KPC-50 also conferred
93 high-level resistance to ceftazidime, it additionally conferred high-level of resistance to CZA
94 (Table 1). Noteworthy, KPC-50 conferred much lower level of resistance to cefoxitin,
95 cefotaxime and cefepime than KPC-3. One of the most marked features of KPC-50 as
96 compared to KPC-3 was that its production did not lead to resistance to carbapenems (Table
97 1). Indeed, *E. coli* expressing the bla_{KPC-50} gene remained susceptible to ertapenem and
98 meropenem (MICs at 0.25 µg/ml and 0.5 µg/ml, respectively), while the MIC of imipenem
99 observed for that KPC-50-producing *E. coli* recombinant strain was at 4 µg/ml (breakpoint
100 value). Low MICs were observed when testing the new carbapenem /ß-lactamase inhibitor
101 combinations such as imipenem/relabactam, and more specifically meropenem/vaborbactam
102 that showed an excellent capacity to inhibit the growth of KPC-50 producers (Table 1).

103 The enzymatic properties of KPC-50 were determined using purified extracts, and
104 compared to those of KPC-3 previously obtained under same conditions (13). Kinetic data
105 showed that KPC-50 has a lower hydrolysis activity of cefalotin, cefotaxime, aztreonam and
106 imipenem, as compared to that of KPC-3 (Table 2). Similar decreased hydrolytic properties
107 toward ß-lactams have been previously reported for those KPC variants conferring resistance

to the CZA combination, such as for KPC-41 (13) or the Asp179Tyr KPC-2 mutants (21-23). Furthermore, the activity of KPC-50 toward aztreonam was not detectable by contrast to KPC-3 (Table 2), and contrasting also with data obtained for KPC-41 (13).

Kinetic activities toward ceftazidime were measured and compared for KPC-50 and KPC-3 enzymes. As expected, a significant hydrolysis rate was detected with KPC-3, but no hydrolysis could be detected with KPC-50 in normal conditions (measurement made during 5 min). Then, another assay was performed during 1 hour, showing that ceftazidime was indeed hydrolyzed by KPC-50, but the hydrolysis rate was much lower than that of KPC-3 (Figure). Therefore, we observed here a paradoxical situation with KPC-50 conferring high-level resistance to ceftazidime once produced by a recombinant *E. coli* clone, but weak hydrolysis rate measured by UV spectrophotometry. Thus, compared affinities of KPC-50 and KPC-3 to the ceftazidime substrate were respectively measured using various concentrations of ceftazidime, to inhibit the hydrolysis of a reporter substrate (nitrocefin) as published (13, 24). At the same ceftazidime concentrations, a higher inhibition level of nitrocefin was observed with KPC-50 than with KPC-3 (Figure), showing that KPC-50 exhibited a higher affinity toward ceftazidime compared to KPC-3.

Comparative inhibitory activity of clavulanic acid, tazobactam and AVI were determined for KPC-50 and KPC-3, showing a 4-fold lower inhibitory activity of AVI toward KPC-50 compared to KPC-3, while conversely that of tazobactam and clavulanic acid were higher toward KPC-50 than KPC-3 (Table 2).

Those results overall indicated that the 276-Glu-Ala-Val-277 insertion observed in the KPC-50 sequence wa responsible for a reduced hydrolysis of cefalotin, cefotaxime, and carbapenems, associated to a higher affinity toward ceftazidime and a reduced sensitivity to AVI.

CONCLUSION

A novel KPC-type enzyme conferring resistance to CZA was identified here from a multidrug-resistant *K. pneumoniae* isolate recovered in Switzerland but likely acquired in Greece, with no known history of treatment with CZA for the patient. Of note, and as already highlighted for KPC-41 and other KPC mutants conferring resistance to CZA, the overall decreased carbapenemase activity observed for KPC-50 might be considered as good news. Furthermore, the newly-developed carbapenem/ß-lactamase inhibitor combinations (meropenem/vaborbactam and imipenem/relabactam) also showed an excellent efficacy

against the KPC-50 producers (either the *K. pneumoniae* clinical isolate or the *E. coli* recombinant strain).

The sequence of KPC-50 has been deposited in the NCBI database under the accession number GenBank MN654342.

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AUTHORS CONTRIBUTIONS

LP and PN designed the study. RZ and SM provided the material. UBG and ST provided the clinical data. XV, MJ and AM performed the experiments. LP and PN wrote the manuscript.

FIGURE LEGEND

Figure. Analysis of ceftazidime hydrolysis. (A) KPC-50 and KPC-3 (1 µM enzyme) hydrolysis of 25 µM ceftazidime (CAZ) at room temperature. (B) and (C) Competitive inhibition curves determined with 50 µM nitrocefin and increasing concentrations of CAZ

157 with 0.1 μM KPC-50 (B) and 0.1 μM KPC-3 (C) at room temperature. For (B) and (C)

158 nitrocefin absorbance was measured.

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Table 1. MICs of β-lactams for *K. pneumoniae* clinical isolate N859, *E. coli* TOP10 recombinant strains producing KPC-50 and KPC-3, respectively, and *E. coli* TOP10 recipient strain

ß-lactams*	MICs (µg/ml)			
	K. pneumoniae N859	*E. coli* TOP10 (pTOPO-KPC-50)	*E. coli* (pTOPO-KPC-3)	*E. coli* TOP10
Amoxicillin	>1,024	>256	>128	8
Amoxicillin – clavulanic acid	>1,024	64	>128	8
Ticarcillin	>1,024	>256	>128	8
Ticarcillin – clavulanic acid	>1,024	64	>128	8
Piperacillin	>1,024	256	>128	2
Piperacillin – tazobactam	>1,024	128	>128	2
Cefalotin	>1,024	512	>128	16
Cefotaxime	8	8	>128	< 0.125
Cefepime	8	8	128	<0.125
Ceftazidime	2,048	1,024	2,048	0.25
Ceftazidime – avibactam	>256	64	2	0.25
Ceftolozane – tazobactam	>256	64	>256	0.06
Cefoxitin	32	8	128	8
Aztreonam	32	16	64	<0.125
Ertapenem	1	0.25	32	<0.125
Imipenem	16	4	16	<0.125
Imipenem - relabactam	2	0.5	0.5	<0.125
Meropenem	2	0.5	16	<0.125
Meropenem - vaborbactam	<0.125	<0.125	0.5	<0.125

*Clavulanic acid was added at a fixed of 2 µg/ml, tazobactam at 4 µg/ml, avibactam at 4 µg/ml, relabactam at 4 µg/ml, and vaborbactam at 8 µg/ml.

Table 2. Kinetic parameters of purified β-lactamases KPC-50 and KPC-3. Data for KPC-3 correspond to those previously reported (13). Inhibitory concentration 50% (IC_{50}) and kinetic inhibition parameters of β-lactamase inhibitors against KPC-50 and KPC-3 (bottom).

	KPC-50			KPC-3		
β-Lactam(s)*	k_{cat} (s-1)	K_m (μM)	k_{cat}/K_m ($mM^{-1}.s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1}.s^{-1}$)
Benzylpenicillin	<0.01	ND	ND	5.6	33	0.2
Cefalotin	2.2	30	0.07	47	113.5	0.4
Cefotaxime	1.7	55	0.003	34.9	532.8	0.065
Ceftazidime	<0.01	ND	ND	> 3.3	> 700	> 4.7 E-3
Aztreonam	<0.01	ND	ND	5	194.8	0.03
Imipenem	2	85	0.02	4.7	71.5	0.07
Meropenem	<0.01	ND	ND	0.47	18.5	0.03
Ertapenem	<0.01	ND	ND	0.58	37	0.02

	IC_{50} (μM)		K_i (μM)	
Inhibitor	KPC-50	KPC-3	KPC-50	KPC-3
Clavulanic acid	10	20	4	20
Tazobactam	10	50	1	10
Avibactam	4	1	2	1

ND : Not determinable due to a low initial rate of hydrolysis
k_{cat}, turnover ; K_m, Michaelis constant (affinity), k_{cat}/K_M = specificity constant (hydrolysis)
IC_{50} represents the concentration of a drug that is required for 50% inhibition of the enzymatic activity
K_i corresponds to a relative k_{off}/k_{on} to the inhibitor for the enzyme

Figure